EVALUATION OF IN VITRO ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF BIOMOLECULES: HYDROXAMIC ACIDS

INTRODUCTION

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibiting other oxidation reactions (1).

History

As part of their adaptation from marine life, terrestrial plant began producing non-marine anti-oxidants such as ascorbic acid (Vitamin C), polyphenols and tocopherol. The evolution of angiosperm plant between 50 and 200 million year ago resulted in the development of many antioxidant pigments – particularly during the Jurassic period-as chemical defenses against reactive oxygen species that are by product of photosynthesis. The term antioxidant originally was used to refer specifically to a chemical that prevents the consumption of oxygen. In the late 19^{th} and early 20^{th} centuries, extensive study was devoted to the uses of antioxidant in important industrial processes, such as the prevention of metal corrosion, the vulcanization of rubber and the polymerization of fuel in the fouling of internal combustion engines (2).

Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fat, which is caused of rancidity. Antioxidant activity could be measured simply by placing the fat in closed container with oxygen and measuring the rate of oxygen consumption (3, 4).

Free Radical

Free radicals are atoms or group of atoms that contain an odd number of electrons and are extremely reactive. These are capable of engaging in rapid chain reaction that destabilize other molecules and generate many more radicals like Reactive Oxygen Species (ROS) which includes

the hydroxyl radical ($OH^{\cdot}$), the super oxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), nitric oxide radical ($NO^{\cdot}$), hypochlorite radical ($ClO^{\cdot}$) and various lipid peroxides (5-10).

Formation of Free Radicals

Normally, bonds would not split in a way that leaves a molecule with odd, unpaired electrons. However, when weak bond splits, free radicals are formed. Free radicals are very unstable and react quickly with other compound trying to gain stability. Generally, free radicals attack the nearest stable molecules abstracting its electron to attain stability. When the attacked molecule loses its electron, it becomes a free radical itself, this formation of free radicals continues and finally resulted in the disruption of the substance especially in fatty foods. Environmental factors such as pollution, radiation, cigarette smoking and herbicides can also spawn free radicals in the body but, if antioxidants are not available to check the free radical production it becomes excessive and cause damage to body and any substance in which oxidation occurs (11, 12). Free radicals are generated largely during the production of ATP in the mitochondria. During this process, radicals coming out from the mitochondria form reactive oxygen species.

Free Radical Scavenger

A free radical scavenger is a molecule that is able to destroy free radicals. Free radicals are very unstable and they move through the bloodstream, taking electrons from other cells or giving away unpaired ones. By doing so, free radicals cause cell damage that has been linked to a host of diseases including heart disease and cancer. The role of the free radical scavenger is to hunt down these unstable molecules and destroy them before they can cause significant cell damage within the body (13).

The free radical scavenger is often referred to as an antioxidant. These scavengers work by preventing the oxidation process that is required in order for electrons to be passed from one cell to another. The free radical scavenger removes the cells or prevents them from oxidizing simply by being oxidized itself within a close proximity. A body without enough free radical scavengers is at greater risk for housing free radicals and for developing serious diseases later in life.

Mechanism of Radical Scavening

- Free radical scavenging antioxidants (AH) inhibit oxidation via chain terminating reactions.
- These have reactive -OH or -NH group.
- Inhibition occurs via a transfer of proton to the free radical species.

$$R^{\cdot} + AH \longrightarrow RH + A^{\cdot} \quad [1]$$

$$RO^{\cdot} + AH \longrightarrow ROH + A^{\cdot} \quad [2]$$

$$ROO^{\cdot} + AH \longrightarrow ROOH + A^{\cdot} \quad [3]$$

- The antioxidant free radical may further interfere with chain propagation reaction by forming peroxy antioxidant compounds.

$$R^{\cdot} + A^{\cdot} \longrightarrow RA \quad [4]$$

$$RO^{\cdot} + A^{\cdot} \longrightarrow ROA \quad [5]$$

$$ROO^{\cdot} + A^{\cdot} \longrightarrow ROOA \quad [6]$$

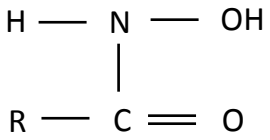
$$\text{Antioxidant} + O_2 \longrightarrow \text{Oxidised antioxidant} \quad [7]$$

Radicals are produced through inflammation, the normal body process by which the body attacks foreign invaders and repairs itself. The body has a built-in ability to eventually neutralize these compounds, once they have done their work. When inflammation persists, the body's own antioxidants may become depleted, allowing reactive oxygen species to accumulate and damage normal healthy cells (14-16). Antioxidants from outside sources can be prescribed to help treat and prevent these diseases.

Hydroxamic Acid as Biomolecules

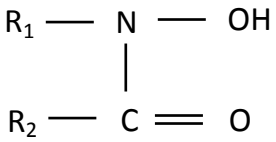
Hydroxamic acids, the neutral polyfunctional molecules were first reported by H. Lossen (17) in 1869. These weak acids, whether naturally occurring or synthetic, constitute one of the most important families of organic bioligands. Hydroxamic acid show a wide spectrum of activities in analytical (18, 19), agricultural (20, 21), biological (22) and medicinal fields (23, 24).

Hydroxamic acids have general formula $R_1NOH.R_2C=O$ (where R_1 and R_2 are phenyl or substituted phenyl group) are naturally occurring or synthetic substances behaving as non electrolytes. In general hydroxamic acids are the N-aryl derivatives of hydroxylamine as shown in figure [I],



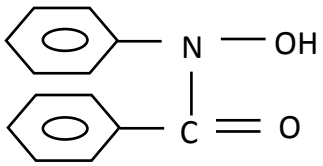
[I]

By replacing the H-atom attached with nitrogen, by alkyl, aryl or cyclic group gives N-Aryl substituted hydroxamic acids [II],



[II]

Bamberger in 1919 (25) laid the foundation of N-arylhydroxamic acids by synthesizing the parent compound of this series, N-phenylhydroxamic acid, trivially named as PBHA [III].



[III]

After recognition of analytical potentialities of PBHA by Shome (26), a large number of analogues of PBHA have been synthesized and reported.

A BRIEF REVIEW OF THE WORK ALREADY DONE IN THIS FIELD

Shimoni et al. (1998) studied antioxidant properties of desferrioxamine E, a new hydroxamate antioxidant in various concentrations and compared to that of desferrioxamine B and BHA (27). Yang et al. (2004) studied that pectin hydroxamic acids exhibit antioxidant activity in vitro as well as protection against hydroxyl radical-mediated DNA damage and anti-human low-density lipoprotein peroxidation tests. The antioxidant effects of BHA were reported to be significantly higher than that of both desferrioxamines (28). Liu et al. (2008) studied antioxidant and nitric oxide production inhibitory activities of galacturonyl hydroxamic acid on LPS-induced NO production in macrophage (29). Koncic et al. (2009) studied antioxidant activity of nonsteroidal anti-inflammatory (NSAIDs) hydroxamic acids and reported significant antioxidant, radical scavenging and metal chelating activities in seven hydroxamic acid derivatives (30). Koncic et al. (2011) reported the antiradical, chelating and antioxidant activities of a series of hydroxyurea derivatives of L- and D-amino acid amides and cycloalkyl-N-arylhydroxamic acids (30).

OBJECTIVES

Hydroxamic acids have attracted considerable attention due to their pharmacological, toxicological and pathological properties over the past decade (31-34). They contain a pharmacophoric structural part and Hydrogen Bond Donor (HBD) and Hydrogen Bond Acceptor (HBA) capability to bind with receptors. It has also been proved that the bioactive molecule attack directly to DNA and kill the tumor/cancer cells (35-40). One of the current approaches in rational drug design is to estimate antioxidant property and DNA binding interaction because these properties of the molecules play an important role in the mechanism of their biological action. **The aim of present investigation is to determine the antioxidant properties of few N-Arylhydroxamic acids.**

NOTEWORTHY CONTRIBUTION IN THE FIELD OF PROPOSED WORK

N-Aryl derivatives of hydroxamic acids for the present investigations are drug like molecules. -NOH.C=O functionality shows both HBD and HBA capability, and this characteristic feature of the molecule is responsible for their medicinal properties. These were also found to posses metal chelating properties (41). Work on QSAR parameters and anti-tumor /anti-cancer activity of some of the N-arylhydroxamic acids were reported from this laboratory (42, 43). The antioxidant activity of parent compound N-phenoxybenzohydroxamic acid already been reported from this laboratory (44).

PROPOSED METHODOLOGY

The following derivatives of N-arylhydroxamic acids will be studied in the present investigation,

1. **N-p-TOLYL-4-ETHOXYBENZOHYDROXAMIC ACID**
2. **N-o- TOLYL-4-ETHOXYBENZOHYDROXAMIC ACID**
3. **N-m-CHLORO-2-METHOXYBENZOHYDROXAMIC ACID**
4. **N-m- CHLORO-4-METHOXYBENZOHYDROXAMIC ACID**
5. **N-PHENYL-4- ETHOXYBENZOHYDROXAMIC ACID**

DETERMINATION OF ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY

The following four methods will be applied to measure the antioxidant property

1. DPPH Method
2. β-Carotene-Linoleate Method
3. Ferrous Thiocyanate Method
4. DNA Cleavage Method

(1) DPPH Method

This is the most widely reported method for screening of antioxidant activity of many plant drugs. DPPH (1,1-diphenyl-2-picryl-hydrazyl) assay method is based on the reduction of ethanolic solution of colored free radical DPPH by free radical scavenger. The procedure involves measurement of decrease in absorbance of DPPH at its absorption maxima of 517 nm, which is proportional to concentration of free radical scavenger added to DPPH reagent solution. The activity is expressed as effective concentration, EC_{50} (45-47). The percent inhibition will be calculated from the following equation [8]:

$$\% \text{ Inhibition} = [(A_0 - A_s / A_0) * 100] \quad [8]$$

where,

A_0 = Absorbance of the blank solution,

A_s = Absorbance of the sample solution.

EC_{50} (Effective Concentration Value)

One parameter that has been introduced recently for the interpretation of the results from the DPPH method, is the "effective concentration" or EC_{50} value (also called the Inhibition Concentration, IC_{50} value).This is defined as the concentration of substrate that causes 50% loss of the DPPH activity (colour). This parameter was apparently introduced by Brand-Williams and his colleagues (48, 49).

(2) β -Carotene Linoleate Method

Oxidation of an aqueous emulsion of β-carotene and linoleic acid was employed as a test for measuring total antioxidant activity of the hydroxamic acids. In this particular model, heat induces formation of linoleic acid free radical. The presence of an antioxidant can reduce the extent of β-carotene destruction by reacting with the linoleate free radical or any other free radical formed within the system. Antioxidant activity will measured as the percentage of inhibition of lipid peroxidation in the β-carotene-linoleic acid system. According to the literature

data (50, 54), normalized antioxidant activity at 60 and 120 minutes of incubation (AA_{60} and AA_{120}) reflects the antioxidant activity more accurately.

The rate of β-carotene bleaching for sample will be calculated according to first-order kinetics [9]:

$$\mathbf{R = 1/t \times \ln A_0/A_t} \qquad \textbf{[9]}$$

where,

t = time in minutes

A_0 = initial absorbance

A_t = absorbance after time interval, t (t = 15, 30, 45 minutes…etc.)

Based on the rates determined at three time intervals, the average rate of β-carotene degradation also be calculated. Furthermore, the antioxidant activity will be calculated from the absolute changes in absorbance at t = 60 and 120 min (AA_{60} and AA_{120}, respectively). Antioxidant activity will be measured as a percentage of inhibition of lipid peroxidation [10].

$$\mathbf{AA = \left[1 - \frac{R_t}{R_0}\right] x\ 100} \qquad \textbf{[10]}$$

where,

R_t = rate of inhibition with sample,

R_0 = control rate of inhibition.

(3) Ferrous Thiocyanate Method

The effect of compounds on the oxidation of linoelic acid using thiocynate method will be studied. The autoxidation of linoelic acid increase with increase in peroxide value. The difference in peroxide value can be found between the control (without antioxidant) and the

linoelic acid containing compound will be studied to find out the antioxidant activity of compound. (55-57).

(4) DNA Cleavage Method

Protective effect of on H_2O_2 induced DNA damage

Electrophoresis is the movement of a charged particle in an electrical field which enables the sorting of molecules based on size and charge. Gel electrophoresis refers to using a gel as an anticonvective medium during electrophoresis. Free radical could damage macromolecules in cells, such as DNA, protein and lipids in membranes. Hydroxy radical causes damage to the Ct-DNA treated with H_2O_2, antioxidant protect these macromolecules from hydroxyl radical which is formed by cleavage of H_2O_2 by donating proton. The DNA molecules can be made to move through the agarose gel and stand with suitable dyes. The results will be analysed by visualizing the gel with UV transilluminator (58-60).

EXPECTED OUTCOME OF THE PROPOSED WORK

Antioxidants is any compound that protects against cellular damage from reactive oxygen species including free radicals, single oxygen species and hydrogen peroxide, which is feature of a wide variety of stubborn health problems in living beings. The present work is an extension of previous work carried on parent compound of N-arylbenzohydroxamic acid series, PBHA. In present investigation the other N-aryl substituted hydroxamic acid will be used to evaluate their antioxidant property.

Hydroxamic acids are drug like molecules which are reported as anti-tumor/anti-cancer agents from this laboratory. The knowledge of their antioxidant property will be helpful to design a better product. The therapeutic strategy to kill cancer cells by reactive oxygen species-mediated mechanism offers promise in a variety of clinical setting and should be considered particularly in the salvage situation.

REFERENCES

1. Sies; Helmut; Exp. physiol., **82**, 291–295 (1997).
2. Knight, J. A.; Ann. clin. & lab. sci., **28**, 331–346 (1998).
3. Pawar, S. M.; Somasekar, I. T., Lakshmi, T. M., Vaithilingam, A., Netare, C.; Reddy, E. P.; J. Pharm. & Biomed. Sci., **4**, 1-4 (2011).
4. Bjelakovic, G.; Syst. Rev. & Meta-Ana., **297**, 842–857 (2007).
5. Wanasundra, P. K. J. P. D.; Shahidi, F.; Bailey's Industrial Oil and Fat Product, Six Edition, John Wiley & Sons., Inc 2005.
6. Dizdaroglu, M.; Free Rad. Biol. Med., **10**, 225–242 (1991).
7. Hurrell, R.; J. Nutr., **133**, 2973S–2977S (2003).
8. Scheibmeir, H. D.; Christensen, K.; Whitaker, S. H.; Jegethesan, J.; Claney, R.; Pierce, J. D.; Int. and Crit. Care Nurs., **21**, 24-28 (2005).
9. Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T. D.; Mazur, M.; Telser, J.; The Inter. J. Biochem. & Cell Bio., **39**, 44-84 (2007).
10. Chapple, I. L. C.; Clin. Pathol. Mol Pathol, **49**, M247-M255 (1996).
11. Borek, C.; The baby-boomer's guide New Canaan connecticus keats publishing, **4**, 51-61 (1991).
12. Pande, R., Khare, D.; Verma, B., Lambert Academic Publisher, Germany (2013).
13. Saha, M. R.; Hasan, S. M. R.; Akter, R.; Hossian, M. M.; Alam, M. S.; Alam, M. S.; Mazumer, M. E. H.; Bangl. J. Vet. Med. , 6 (2), 197-202 (2008).
14. Koncic, M. Z.; Rajic, Z.; Petric, N.; Zorc, B.; Acta Pharm., **59**, 235-242 (2009).
15. Yang, S. S.; Cheng, K. D.; Lin, Y. S.; Liu, Y. W.; Hou, W. C.; J. Agri. Food Chem., **52**, 4270–4273 (2004).
16. Shimonia, E.; Zahnerb, H.; Neemana, I.; JAOCS, **75**, 1453-1455 (1998).
17. Lossen, H.; Ann.Chem., **150**, 314 (1869).
18. Hassan, K. F.; Kandil, S. A.; Abdel Aziz, H. M.; Siyam, T.; Chrom. Res. Int., **2011** (2011).
19. Fraizer, C. P.; Engelking, J. R.; Aianiz, J. R. D.; J. Am. Chem. Soc., **133,** 10430-10433 (2011).

20. Dimkpa, C.; Svatos, A.; Merten, D.; Buchel, G.; Kothe, E.; Can. J. Microbiol., **54**, 163 (2008).
21. Bravo, H.R.; villarroel, E.; Copja, S.V.; Argandona, V. H.; Naturforsch, Z.; **63**, 389 (2008).
22. Hoidy, W. H.; Ahmad, M.B.; Emad, A.; Al-Mulla, J.; Yunus, W. M. Z. W., Bt Ibrahim, N. A.; J. Olea Sci., **59**, 15-19 (2010).
23. Kwak, S. Y.; Lee, S.; Choi, H. R.; Park, K. C.; Bio. Med. Chem. Let., **21**, 5155-5158 (2011).
24. Oanh, D. T. K.; Hai, H. V.; Park, S. H.; Kim, H. J.; Han, B. W.; Kim, H. S.; Hong, J. T.; Han, S. B.; Hue, V. T. M.; Nam, N. H.; Bio. Med. Chem. Let., **21**, 7509-7512 (2011).
25. Bamberger, E.; Ber., **52**, 116 (1919).
26. Shome, S.C.; Analyst, **75**, 27 (1950).
27. Shimonia, E.; Zahnerb, H.; Neemana, I.; JAOCS, **75**, 1453-1455 (1998).
28. Yang, S. S.; Cheng, K. D.; Lin, Y. S.; Liu, Y. W.; Hou, W. C.; J. Agri. Food Chem., **52**, 4270–4273 (2004).
29. Liu , Y. H.; Lin, S. Y.; Lee,C. C.; Hau; W. C.; Food Chem., **109**, 159-166 (2008).
30. Koncic, M. Z.; Rajic, Z.; Petric, N.; Zorc, B.; Acta Pharm., **59**, 235-242 (2009).
31. Fytas, C.; Zoidis, G.; Tzoutzas, N.; Taylor, M. C.; Fytas, G.; Kelly, J. M.; J. Med. Chem., **54**, 5250 (2011).
32. Shanker, S.; Bernstein, M.; Momparter, L. F.; Momparter, R.L.; *Leuk. Res*., **27**, 437 (2003).
33. Jackman, J.E.; Fierke, C.A.; Tumey, L.N.; Pirrung, M.; Uchiyama, T.; Tahir, S.H.; Hindsgaul, O.; Raetz, C.R.; *J. Biol. Chem*., **275**, 11002 (2000).
34. Bravo, H.R.; Villarroel, E.; Copaja, S.V.; Argandona, V.H.; *Z Naturforsh*, **63**, 389 (2008).
35. Koncic, M. Z.; Barbaric, M.; Perkovic, I.; Zorc, B.; Molecules, **16**, 6232-6242 (2011).
36. Zhang, Y.; Feng, J.; Jia,Y.; Wang, X.; Zhang, L.; Liu, C.; Fang, H.; and Xu, W.; J. Med. Chem. **54** 2823 (2011).
37. Vigushin, D.M.; Ali, S.; Pace, P.E.; Mirsaido, N.; Ito, K.; Adcock, I.; Coombus, R.G.; Clin. Cancer Res., **7**, 971 (2001).

38. Noh, E.J.; Lim, D.S.; Jeong, G.; Lee, J.S.; Biochemical and Biophysical Research Communications, **378**, 326 (2009).
39. Zhang, Y.; Feng, J.; Liu, C.; Fang H.; Xu, W.; Bioorg. & Med. Chem., **19** 4437 (2011).
40. Hoffman, H.E.; Jiraskova, J.; Cigler, P.; Sanda, M.; Schraml, J.; Konvalinka, J.; J. Med. Chem., **52**, 6032 (2009).
41. Carrott, M.J.; Fox, O.D.; Maher, C.J.; Mason, C.; Taylor, R.J.; Snikov, S.I.; Choppin, G. R.; Sol. Extr. Ion Exch., **25**, 723-746 (2007).
42. Rajwade, R.P.; Pande, R.; Mishra, K. P; Kumar, A.; Pandey, B.N., Med. Chem., Bentham Sci., **4**, 237 (2008).
43. Rajwade, R.P.; Pande, R.; Ana. Chem. Acta., **630**, 205 (2008).
44. Khare, D.; Verma, B.; Pande, R.; Asian J. Pharm. Clin. Res., **5(4)**, 121-124 (2012).
45. Vani, T.; Rajani, M.; Sarkar, S.; Shishoo, C. J.; Inter. J. Pharmacognosy, **35**, 313-317 (1997).
46. Sanchez-Moreno, C.; Larrauri, J.; Saura-Calixto, F.; J. Sci.Food. Agric., **79**, 1301-1304 (1999).
47. Navarro, M. C; **59**, 312-314 (1993).
48. Brand-Williams, W.; Cuvelier, M., E.; Berset, C.; Food Sci. & Tech., **28**, 25-30 (1995).
49. Bondet, V.; Brand-Williams, W.; Berset, C.; Food Sci. & Tech., **30**, 609-615 (1997).
50. Al-Saikhan, M. S.; Howard, L. R.; Miller Jr, J. C.; J. Food Sci., **60**, 341-343 (1995).
51. Amarowicz, R.; Pegg, R., B.; Rahimi-Moghaddam, P.; Barl, B., Weil, J. A.; Food Chem., **84**, 551-562 (2004).
52. Sarikurkcu, C.; Arisoy, K.; Tepe, B.; Cakir, A.; Abali, G.; Food Chem. Toxicol., **47**, 2479 (2009).
53. Al-Saikhan, M. S.; Howard, L. R.; Miller Jr, J. C.; J. Food Sci., **60**, 341-343 (1995).
54. Koncic, M. Z.; Rajic, Z.; Petric, N.; Zorc, B.; Acta Pharm., **59**, 235-242 (2009).
55. Nakatani, N.; Kikuzaki, H.; Agric. Biol. Chem., **51**, 2727-2732 (1987).
56. Ali Ebrahimzadeh, M,; Nabavi, S. M.; Nabavi, S., F.; Bahramian, F.; Bekhradnia, A. R., Pak. J. Pharm. Sci., **23**, 29-34 (2012).
57. Rezaeizadeh, A.; Zuki, A.; Abdollahi, M.; Goh, Y. M.; Noordin, M. M.; Hamid, M.; Azmi, T.; African Journal of Biotechnology, **10**, 4932-4940 (2011).

58. Attaguile, G.; Russo, A., Campisi, A.; Savoca, F.; Acquaviva, R.; Ragusa, N.; Vanella, A.; Cell Bio. & Toxicol., **16**, 83-90 (2000).

59. Xua Z-D.; Liub, H.; Wanga, M.; Xiaoa, S-L.; Yanga, M.; bub, X-H.; J. Inorg. Biochem., **92**, 149 (2002).

60. Khan,S.N.; Danishuddin, M.; Khan, A.U.; Biosci. Rep., **30**, 331 (2010).

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